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(54) Title: HUMAN NEURONAL NICOTINIC ACETYLCHOLINE RECEPTOR COMPOSITIONS AND METHODS EMPLOYING SAME

(57) Abstract

DNA encoding human neuronal nicotinic acetylcholine receptor α_2 subunits, mammalian and amphibian cells containing said DNA, and methods for producing such subunits are provided. In addition, combinations of subunits (i.e., α_2, plus α_1, α_3, α_4, and/or α_7 subunits in combination with β_2, β_3 and/or β_4 subunits) are provided.

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HUMAN NEURONAL NICOTINIC ACETYLCHOLINE RECEPTOR COMPOSITIONS AND METHODS EMPLOYING SAME

RELATED APPLICATIONS

This application is related to United States Serial Nos. 07/504,455, filed April 3, 1990, now pending; 07/938,154, filed November 30, 1992, now pending; and 08/028,031, filed March 8, 1993, now pending.

FIELD OF INVENTION

This invention relates to nucleic acids encoding human neuronal nicotinic acetylcholine receptor protein subunits, as well as the proteins themselves. In particular, nucleic acids encoding human neuronal nicotinic acetylcholine receptor alpha subunits and beta subunits, and alpha subunit proteins, beta subunit proteins, and combinations thereof are provided.

BACKGROUND OF THE INVENTION

Ligand-gated ion channels provide a means for communication between cells of the central nervous system. These channels convert a signal (e.g., a chemical referred to as a neurotransmitter) that is released by one cell into an electrical signal that propagates along a target cell membrane. A variety of neurotransmitters and neurotransmitter receptors exist in the central and peripheral nervous systems. Five families of ligand-gated receptors, including the nicotinic acetylcholine receptors (NAChRs) of neuromuscular and neuronal origins, have been identified (Stroud et al. (1990) Biochemistry 29:11009-11023). There is, however, little understanding of the manner in which the variety of receptors generates different responses to neurotransmitters or to other modulating ligands in different regions of the nervous system.

The nicotinic acetylcholine receptors (NAChRs) are multisubunit proteins of neuromuscular and neuronal origins. These receptors form ligand-gated ion channels that mediate synaptic transmission between nerve and muscle and between neurons upon interaction with the neurotransmitter acetylcholine (ACh). Since various nicotinic acetylcholine receptor (NAChR) subunits exist, a variety of NAChR compositions (i.e., combinations of subunits) exist. The different NAChR compositions exhibit different specificities for various ligands and are thereby pharmacologically distinguishable. Thus, the nicotinic acetylcholine receptors expressed at the vertebrate neuromuscular junction in vertebrate sympathetic ganglia and in the vertebrate central nervous system have been distinguished on the basis of the effects of various ligands that bind to different NAChR compositions. For example, the elapid α-neurotoxins that block activation of nicotinic acetylcholine receptors at the neuromuscular junction do not block activation of some neuronal nicotinic acetylcholine receptors that are expressed on several different neuron-derived cell lines.

Muscle NAChR is a glycoprotein composed of five subunits with the stoichiometry $\alpha_2\beta(\gamma \text{ or } \epsilon)\delta$. Each of the subunits has a mass of about 50-60 kilodaltons (kd) and is encoded by a different gene. The $\alpha_2\beta(\gamma \text{ or } \epsilon)\delta$ complex forms functional receptors containing two ligand binding sites and a ligand-gated transmembrane channel. Upon interaction with a cholinergic agonist, muscle nicotinic AChRs conduct sodium ions. The influx of sodium ions rapidly short-circuits the normal ionic gradient maintained across the plasma membrane, thereby depolarizing the membrane. By reducing the potential difference across the membrane, a chemical signal is transduced into an electrical signal that signals muscle contraction at the neuromuscular junction.

Functional muscle nicotinic acetylcholine receptors have been formed with $\alpha\beta\delta\gamma$ subunits, $\alpha\beta\gamma$ subunits, $\alpha\beta\delta$ subunits, $\alpha\delta\gamma$ subunits or $\alpha\delta$ subunits, but not with only one subunit (see e.g., Kurosaki et al. (1987) FEBS Lett. 214: 253-258; Camacho et al. (1993) J. Neuroscience 13:605-613). In contrast, functional neuronal AChRs (nAChRs) can be formed from α subunits alone or combinations of α and β subunits. The larger α subunit is generally believed to be the ACh-binding subunit and the lower molecular weight β subunit is generally believed to be the structural subunit, although it has not been definitively demonstrated that the β subunit does not have the ability to bind ACh. Each of the subunits which participate in the formation of a functional ion channel are, to the extent they contribute to the structure of the resulting channel, "structural" subunits, regardless of their ability (or inability) to bind ACh. Neuronal AChRs (nAChRs), which are also ligand-gated ion channels, are expressed in ganglia of the autonomic nervous system and in the central nervous system (where they mediate signal transmission), in post-synaptic locations (where they modulate transmission), and in pre- and extra-synaptic locations (where they may have additional functions).

DNA encoding NAChRs has been isolated from several sources. Based on the information available from such work, it has been evident for some time that NAChRs expressed in muscle, in autonomic ganglia, and in the central nervous system are functionally diverse. This functional diversity could be due, at least in part, to the large number of different NAChR subunits which exist. There is an incomplete understanding, however, of how (and which) NAChR subunits combine to generate unique NAChR subtypes, particularly in neuronal cells. Indeed, there is evidence that only certain NAChR subtypes may be involved in diseases such as Alzheimer's disease.

Moreover, it is not clear whether NAChRs from analogous tissues or cell types are similar across species.

Accordingly, there is a need for the isolation and characterization of nucleic acids encoding each human neuronal NAChR subunit, recombinant cells containing such subunits and receptors prepared therefrom. In order to study the function of human neuronal AChRs and to obtain disease-specific pharmacologically active agents, there is also a need to obtain isolated (preferably purified) human neuronal nicotinic AChRs, and isolated (preferably purified) human neuronal nicotinic AChR subunits. In addition, there is also a need to develop assays to identify such pharmacologically active agents.

The availability of such nucleic acids, cells, receptor subunits and receptor compositions will eliminate the uncertainty of speculating as to human nNAChR structure and function based on predictions drawn from non-human nNAChR data, or human or non-human muscle or ganglia NAChR data.

Therefore, it is an object herein to isolate and characterize nucleic acids encoding subunits of human neuronal nicotinic acetylcholine receptors. It is also an object herein to provide methods for recombinant production of human neuronal nicotinic acetylcholine receptor subunits. It is also an object herein to provide purified receptor subunits and to provide methods for screening compounds to identify compounds that modulate the activity of human neuronal AChRs.

These and other objects will become apparent to those of skill in the art upon further study of the specification and claims.

BRIEF DESCRIPTION OF THE INVENTION

In accordance with the present invention, there are provided isolated nucleic acids encoding novel human alpha subunits of neuronal NAChRs. In particular, isolated DNA encoding human α_2 subunits of neuronal NAChRs are provided. Messenger RNA and polypeptides encoded by the above-described nucleic acids are also provided.

Further in accordance with the present invention, there are provided recombinant α_2 subunits of human neuronal nicotinic AChRs, as well as methods for the production thereof. In addition, recombinant neuronal nicotinic acetylcholine receptors containing a human α_2 subunit of neuronal nicotinic AChRs are also provided, as well as methods for the production thereof. Further provided are recombinant neuronal nicotinic AChRs that contain a mixture of one or more NAChR subunits encoded by a host cell, and one or more nNAChR subunits encoded by heterologous DNA or RNA (i.e., DNA or RNA as described herein that has been introduced into the host cell), as well as methods for the production thereof.

Plasmids containing DNA encoding the above-described subunits are also provided. Recombinant cells containing the above-described DNA, mRNA or plasmids are also provided herein. Such cells are useful, for example, for replicating DNA, for producing human NAChR subunits and recombinant receptors, and for producing cells that express receptors containing one or more human subunits.

Also provided in accordance with the present invention are methods for identifying cells that express functional nicotinic acetylcholine receptors. Methods for identifying compounds which modulate the activity of NAChRs are also provided.

The DNA, mRNA, vectors, receptor subunits, receptor subunit combinations and cells provided herein permit production of selected neuronal nicotinic AChR subunits and specific combinations thereof, as well as antibodies to said receptor subunits. This provides a means to prepare synthetic or recombinant receptors and receptor subunits that are substantially free of contamination from many other receptor proteins whose presence can interfere with analysis of a single NAChR subtype. The availability of desired receptor subtypes makes it possible to observe the effect of a drug substance on a particular receptor subtype and to thereby perform initial *in vitro* screening of the drug substance in a test system that is specific for humans and specific for a human neuronal nicotinic AChR subtype.

The availability of subunit-specific antibodies makes possible the application of the technique of immunohistochemistry to monitor the distribution and expression density of various subunits (e.g., in normal vs diseased brain tissue). Such antibodies could also be employed for diagnostic and therapeutic applications.

The ability to screen drug substances *in vitro* to determine the effect of the drug on specific receptor compositions should permit the development and screening of receptor subtype-specific or disease-specific drugs. Also, testing of single receptor subunits or specific receptor subunit combinations with a variety of potential agonists or antagonists provides additional information with respect to the function and activity of the individual subunits and should lead to the identification and design of compounds that are capable of very specific interaction with one or more of the receptor subunits or receptor subtypes. The resulting drugs should exhibit fewer unwanted side effects than drugs identified by screening with cells that express a variety of subtypes.

Further in relation to drug development and therapeutic treatment of various disease states, the availability of nucleic acids encoding human nNAChR subunits enables identification of any alterations in such genes (e.g., mutations) which may correlate with the occurrence of certain disease states. In addition, the creation of animal models of such disease states becomes possible, by specifically introducing such mutations into synthetic DNA sequences which can then be introduced into laboratory animals or *in vitro* assay systems to determine the effects thereof.

BRIEF DESCRIPTION OF THE FIGURE

Figure 1 presents a restriction map of two pCMV promoter-based vectors, pCMV-T7-2 and pCMV-T7-3.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, we have isolated and characterized nucleic acids encoding a novel human alpha subunit of neuronal NAChRs. Specifically, isolated DNAs encoding a human α_2 subunit of neuronal NAChRs are described herein. Recombinant messenger RNA (mRNA) and recombinant polypeptides encoded by the above-described nucleic acids are also provided.

As used herein, isolated (or substantially pure) as a modifier of DNA, RNA, polypeptides or proteins means that the DNA, RNA, polypeptides or proteins so designated have been separated from their *in vivo* cellular environments through the efforts of human beings. Thus as used herein, isolated (or substantially pure) DNA refers to DNAs purified according to standard techniques employed by those skilled in the art (see, e.g., Maniatis et al.(1982) Molecular Cloning: A

Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

Similarly, as used herein, "recombinant" as a modifier of DNA, RNA, polypeptides or proteins means that the DNA, RNA, polypeptides or proteins so designated have been prepared by the efforts of human beings, e.g., by cloning, recombinant expression, and the like. Thus as used herein, recombinant proteins, for example, refers to proteins produced by a recombinant host, expressing DNAs which have been added to that host through the efforts of human beings.

As used herein, a human alpha subunit gene is a gene that encodes an alpha subunit of a human neuronal nicotinic acetylcholine receptor. The alpha subunit is a subunit of the NAChR to which ACh binds. Assignment of the name "alpha" to a putative nNAChR subunit, according to Deneris et al. [Tips (1991) 12:34-40], is based on the conservation of adjacent cysteine residues in the presumed extracellular domain of the subunit that are the homologues of cysteines 192 and 193 of the *Torpedo* alpha subunit (see Noda et al. (1982) Nature 299:793-797). As used herein, an alpha subunit refers to a human nNAChR subunit that is encoded by DNA that hybridizes under high stringency conditions to the nNAChR alpha subunit-encoding DNAs disclosed herein. An alpha subunit also binds to ACh under physiological conditions and at physiological concentrations and, in the optional presence of a beta subunit (i.e., some alpha subunits are functional alone, while others require the presence of a beta subunit), generally forms a functional AChR as assessed by methods described herein or known to those of skill in this art.

As used herein, "α_2 subunit DNA" refers to DNA encoding a neuronal nicotinic acetylcholine receptor

subunit of the same name. Such DNA can be characterized in a number of ways, for example, the nucleotides of said DNA may encode the amino acid sequence set forth in SEQ. ID No. 2. Presently preferred α_2-encoding DNAs can be characterized as DNA which hybridizes under high stringency conditions to the coding sequence set forth in SEQ. ID No. 1 (preferably to substantially the entire coding sequence thereof, i.e., nucleotides 166-1755). Especially preferred α_2-encoding DNAs of the invention are characterized as having substantially the same nucleotide sequence as the coding region set forth in SEQ. ID No. 1 (i.e., nucleotides 166-1755 thereof).

Typically, unless an α_2 subunit arises as a splice variant, α_2-encoding DNA will share substantial sequence homology (i.e., greater than about 90%), with the α_4 DNAs described herein. DNA or RNA encoding a splice variant may share less than 90% overall sequence homology with the DNA or RNA provided herein, but such a splice variant would include regions of nearly 100% homology to the above-described DNAs.

Also contemplated are alpha subunits encoded by DNAs that encode alpha subunits as defined above, but that by virtue of degeneracy of the genetic code do not necessarily hybridize to the disclosed DNA or deposited clones under specified hybridization conditions. Such subunits also contribute to the formation of a functional receptor, as assessed by the methods described herein or known to those of skill in the art, generally with one or more beta subunits. Typically, unless an alpha subunit is encoded by RNA that arises from alternative splicing (i.e., a splice variant), alpha-encoding DNA and the alpha subunit encoded thereby share substantial sequence homology with the alpha subunit DNAs (and proteins encoded thereby) described herein. It is understood that DNA or RNA encoding a splice variant may overall share

less than 90% homology with the DNA or RNA provided herein, but include regions of nearly 100% homology to a DNA fragment described herein, and encode an open reading frame that includes start and stop codons and encodes a functional alpha subunit.

As used herein, a splice variant refers to variant NAChR subunit-encoding nucleic acid(s) produced by differential processing of primary transcript(s) of genomic DNA, resulting in the production of more than one type of mRNA. cDNA derived from differentially processed genomic DNA will encode NAChR subunits that have regions of complete amino acid identity and regions having different amino acid sequences. Thus, the same genomic sequence can lead to the production of multiple, related mRNAs and proteins. Both the resulting mRNAs and proteins are referred to herein as "splice variants".

Stringency of hybridization is used herein to refer to conditions under which polynucleic acid hybrids are stable. As known to those of skill in the art, the stability of hybrids is reflected in the melting temperature (T_m) of the hybrids. T_m can be approximated by the formula:

$$81.5°C - 16.6(\log_{10}[Na^+]) + 0.41(\%G+C) - 600/l,$$

where l is the length of the hybrids in nucleotides. T_m decreases approximately 1-1.5°C with every 1% decrease in sequence homology. In general, the stability of a hybrid is a function of sodium ion concentration and temperature. Typically, the hybridization reaction is performed under conditions of lower stringency, followed by washes of varying, but higher, stringency. Reference to hybridization stringency relates to such washing conditions. Thus, as used herein:

(1) HIGH STRINGENCY refers to conditions that permit hybridization of only those nucleic acid sequences that form stable hybrids in 0.018M NaCl at 65°C (i.e., if a hybrid is not stable in 0.018M NaCl at 65°C, it will not be stable under high stringency conditions, as contemplated herein). High stringency conditions can be provided, for example, by hybridization in 50% formamide, 5X Denhardt's solution, 5X SSPE, 0.2% SDS at 42°C, followed by washing in 0.1X SSPE, and 0.1% SDS at 65°C;

(2) MODERATE STRINGENCY refers to conditions equivalent to hybridization in 50% formamide, 5X Denhardt's solution, 5X SSPE, 0.2% SDS at 42°C, followed by washing in 0.2X SSPE, 0.2% SDS, at 65°C; and

(3) LOW STRINGENCY refers to conditions equivalent to hybridization in 10% formamide, 5X Denhardt's solution, 6X SSPE, 0.2% SDS, followed by washing in 1X SSPE, 0.2% SDS, at 50°C.

It is understood that these conditions may be duplicated using a variety of buffers and temperatures and that they are not necessarily precise.

Denhardt's solution and SSPE (see, e.g., Sambrook, Fritsch, and Maniatis, in: Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1989) are well known to those of skill in the art as are other suitable hybridization buffers. For example, SSPE is pH 7.4 phosphate-buffered 0.18M NaCl. SSPE can be

prepared, for example, as a 20X stock solution by dissolving 175.3 g of NaCl, 27.6 g of NaH_2PO_4 and 7.4 g EDTA in 800 ml of water, adjusting the pH to 7.4, and then adding water to 1 liter. Denhardt's solution (see, Denhardt (1966) Biochem. Biophys. Res. Commun. 23:641) can be prepared, for example, as a 50X stock solution by mixing 5 g Ficoll (Type 400, Pharmacia LKB Biotechnology, INC., Piscataway NJ), 5 g of polyvinylpyrrolidone, 5 g bovine serum albumin (Fraction V; Sigma, St. Louis MO) water to 500 ml and filtering to remove particulate matter.

The phrase "substantial sequence homology" is used herein in reference to the nucleotide sequence of DNA, the ribonucleotide sequence of RNA, or the amino acid sequence of protein, that have slight and non-consequential sequence variations from the actual sequences disclosed herein. Species having substantial sequence homology are considered to be equivalent to the disclosed sequences and as such are within the scope of the appended claims. In this regard, "slight and non-consequential sequence variations" mean that "homologous" sequences, i.e., sequences that have substantial homology with the DNA, RNA, or proteins disclosed and claimed herein, are functionally equivalent to the sequences disclosed and claimed herein. Functionally equivalent sequences will function in substantially the same manner to produce substantially the same compositions as the nucleic acid and amino acid compositions disclosed and claimed herein. In particular, functionally equivalent DNAs encode proteins that are the same as those disclosed herein or that have conservative amino acid variations, such as substitution of a non-polar residue for another non-polar residue or a charged residue for a similarly charged residue. These changes include those recognized by those of skill in the art as those that do not

substantially alter the tertiary structure of the protein.

In practice, the term substantially the same sequence means that DNA or RNA encoding two proteins hybridize under conditions of high stringency and encode proteins that have the same sequence of amino acids or have changes in sequence that do not alter their structure or function. As used herein, substantially identical sequences of nucleotides share at least about 90% identity, and substantially identical amino acid sequences share more than 95% amino acid identity. It is recognized, however, that proteins (and DNA or mRNA encoding such proteins) containing less than the above-described level of homology arising as splice variants or that are modified by conservative amino acid substitutions (or substitution of degenerate codons) are contemplated to be within the scope of the present invention.

DNA encoding human neuronal nicotinic AChR α_2 subunits may be isolated by screening suitable human cDNA or human genomic libraries under suitable hybridization conditions with DNA disclosed herein. Suitable libraries can be prepared from neuronal tissue samples, hippocampus tissue, or cell lines, such as the human neuroblastoma cell line IMR32 (ATCC Accession No. CCL127), and the like. The library is preferably screened with a portion of DNA including the entire subunit-encoding sequence thereof, or the library may be screened with a suitable probe.

As used herein, a probe is single-stranded DNA or RNA that has a sequence of nucleotides that includes at least 14 contiguous bases that are the same as (or the complement of) any 14 bases set forth in SEQ ID No. 1. Preferred regions from which to construct probes include

5' and/or 3' coding sequences, sequences predicted to encode transmembrane domains, sequences predicted to encode the cytoplasmic loop, signal sequences, acetylcholine (ACh) and α-bungarotoxin (α-bgtx) binding sites, and the like. Amino acids 210-220 are typically involved in ACh and α-bgtx binding. The approximate amino acid residues which comprise such regions specifically for α_2 subunits include amino acids 1-55 for the signal sequence, amino acids 264-289 for the first transmembrane domain (TMD1), amino acids 297-320 for the second transmembrane domain (TMD2), amino acids 326-350 for the third transmembrane domain (TMD3), amino acids 444-515 for the fourth transmembrane domain (TMD4), and amino acids 351-443 for the cytoplasmic loop. Alternatively, portions of the DNA can be used as primers to amplify selected fragments in a particular library.

After screening the library, positive clones are identified by detecting a hybridization signal; the identified clones are characterized by restriction enzyme mapping and/or DNA sequence analysis, and then examined, by comparison with the sequences set forth herein, to ascertain whether they include DNA encoding a complete alpha subunit. If the selected clones are incomplete, they may be used to rescreen the same or a different library to obtain overlapping clones. If desired, the library can be rescreened with positive clones until overlapping clones that encode an entire alpha subunit are obtained. If the library is a cDNA library, then the overlapping clones will include an open reading frame. If the library is genomic, then the overlapping clones may include exons and introns. In both instances, complete clones may be identified by comparison with the DNA and encoded proteins provided herein.

Complementary DNA clones encoding human nNAChR α_2 subunits have been isolated. The DNA clones provided

herein may be used to isolate genomic clones encoding such subunits and to isolate any splice variants by screening libraries prepared from different neural tissues. Nucleic acid amplification techniques, which are well known in the art, can be used to locate splice variants of human NAChR subunits. This is accomplished by employing oligonucleotides based on DNA sequences surrounding divergent sequence(s) as primers for amplifying human RNA or genomic DNA. Size and sequence determinations of the amplification products can reveal the existence of splice variants. Furthermore, isolation of human genomic DNA sequences by hybridization can yield DNA containing multiple exons, separated by introns, that correspond to different splice variants of transcripts encoding human NAChR subunits.

The above-described nucleotide sequences can be incorporated into vectors for further manipulation. As used herein, vector (or plasmid) refers to discrete elements that are used to introduce heterologous DNA into cells for either expression or replication thereof. Selection and use of such vehicles are well within the level of skill of the art.

An expression vector includes vectors capable of expressing DNAs that are operatively linked with regulatory sequences, such as promoter regions, that are capable of effecting expression of such DNA fragments. Thus, an expression vector refers to a recombinant DNA or RNA construct, such as a plasmid, a phage, recombinant virus or other vector that, upon introduction into an appropriate host cell, results in expression of the cloned DNA. Appropriate expression vectors are well known to those of skill in the art and include those that are replicable in eukaryotic cells and/or prokaryotic cells and those that remain episomal or those which integrate into the host cell genome. Presently preferred

plasmids for expression of invention AChR subunits in eukaryotic host cells, particularly mammalian cells, include cytomegalovirus (CMV) promoter-containing vectors such as pCMV, pcDNA1, and the like.

As used herein, a promoter region refers to a segment of DNA that controls transcription of DNA to which it is operatively linked. The promoter region includes specific sequences that are sufficient for RNA polymerase recognition, binding and transcription initiation. This portion of the promoter region is referred to as the promoter. In addition, the promoter region includes sequences that modulate this recognition, binding and transcription initiation activity of RNA polymerase. These sequences may be *cis* acting or may be responsive to *trans* acting factors. Promoters, depending upon the nature of the regulation, may be constitutive or regulated. Exemplary promoters contemplated for use in the practice of the present invention include the SV40 early promoter, the cytomegalovirus (CMV) promoter, the mouse mammary tumor virus (MMTV) steroid-inducible promoter, Moloney murine leukemia virus (MMLV) promoter, and the like.

As used herein, the term "operatively linked" refers to the functional relationship of DNA with regulatory and effector sequences of nucleotides, such as promoters, enhancers, transcriptional and translational stop sites, and other signal sequences. For example, operative linkage of DNA to a promoter refers to the physical and functional relationship between the DNA and the promoter such that the transcription of such DNA is initiated from the promoter by an RNA polymerase that specifically recognizes, binds to and transcribes the DNA. In order to optimize expression and/or *in vitro* transcription, it may be necessary to remove or alter 5' untranslated portions of the clones to remove extra,

potential alternative translation initiation (i.e., start) codons or other sequences that interfere with or reduce expression, either at the level of transcription or translation. Alternatively, consensus ribosome binding sites (see, for example, Kozak (1991) J. Biol. Chem. 266:19867-19870) can be inserted immediately 5' of the start codon to enhance expression. The desirability of (or need for) such modification may be empirically determined.

As used herein, expression refers to the process by which polynucleic acids are transcribed into mRNA and translated into peptides, polypeptides, or proteins. If the polynucleic acid is derived from genomic DNA, expression may, if an appropriate eukaryotic host cell or organism is selected, include splicing of the mRNA.

Particularly preferred vectors for transfection of mammalian cells are the pSV2dhfr expression vectors, which contain the SV40 early promoter, mouse dhfr gene, SV40 polyadenylation and splice sites and sequences necessary for maintaining the vector in bacteria, cytomegalovirus (CMV) promoter-based vectors such as pCDNA1 (Invitrogen, San Diego, CA), and MMTV promoter-based vectors such as pMSG (Catalog No. 27-4506-01 from Pharmacia, Piscataway, NJ).

Full-length DNAs encoding human neuronal NAChR subunits have been inserted into vector pCMV-T7, a pUC19-based mammalian cell expression vector containing the CMV promoter/enhancer, SV40 splice/donor sites located immediately downstream of the promoter, a polylinker downstream of the splice/donor sites, followed by an SV40 polyadenylation signal. Placement of NAChR subunit DNA between the CMV promoter and SV40 polyadenylation signal provides for constitutive expression of the foreign DNA

in a mammalian host cell transfected with the construct. For inducible expression of human NAChR subunit-encoding DNA in a mammalian cell, the DNA can be inserted into a plasmid such as pMSG. This plasmid contains the mouse mammary tumor virus (MMTV) promoter for steroid-inducible expression of operatively associated foreign DNA. If the host cell does not express endogenous glucocorticoid receptors required for uptake of glucorcorticoids (i.e., inducers of the MMTV promoter) into the cell, it is necessary to additionally transfect the cell with DNA encoding the glucocorticoid receptor (ATCC accession no. 67200). Full-length human DNA clones encoding human α_2 subunits can also be subcloned into pIBI24 (International Biotechnologies, Inc., New Haven, CT) or pCMV-T7-2 for synthesis of *in vitro* transcripts.

In accordance with another embodiment of the present invention, there are provided cells containing the above-described polynucleic acids (i.e., DNA or mRNA). Such host cells as bacterial, yeast and mammalian cells can be used for replicating DNA and producing nAChR subunit(s). Methods for constructing expression vectors, preparing *in vitro* transcripts, transfecting DNA into mammalian cells, injecting oocytes, and performing electrophysiological and other analyses for assessing receptor expression and function as described herein are also described in PCT Application Nos. PCT/US91/02311, PCT/US91/05625 and PCT/US92/11090, and in co-pending U.S. Application Serial Nos. 07/504,455, 07/563,751 and 07/812,254. The subject matter of these applications are hereby incorporated by reference herein in their entirety.

Incorporation of cloned DNA into a suitable expression vector, transfection of eukaryotic cells with a plasmid vector or a combination of plasmid vectors, each encoding one or more distinct genes or with linear

DNA, and selection of transfected cells are well known in the art (see, e.g., Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press). Heterologous DNA may be introduced into host cells by any method known to those of skill in the art, such as transfection with a vector encoding the heterologous DNA by $CaPO_4$ precipitation (see, e.g., Wigler et al. (1979) Proc. Natl. Acad. Sci. 76:1373-1376). Recombinant cells can then be cultured under conditions whereby the subunit(s) encoded by the DNA is (are) expressed. Preferred cells include mammalian cells (e.g., HEK 293, CHO and Ltk$^-$ cells), yeast cells (e.g., methylotrophic yeast cells, such as *Pichia pastoris*), bacterial cells (e.g., *Escherichia coli*), and the like.

While the DNA provided herein may be expressed in any eukaryotic cell, including yeast cells (such as, for example, *P. pastoris* (see U.S. Patent Nos. 4,882,279, 4,837,148, 4,929,555 and 4,855,231), *Saccharomyces cerevisiae*, *Candida tropicalis*, *Hansenula polymorpha*, and the like), mammalian expression systems, including commercially available systems and other such systems known to those of skill in the art, for expression of DNA encoding the human neuronal nicotinic AChR subunits provided herein are presently preferred. *Xenopus* oocytes are preferred for expression of RNA transcripts of the DNA.

In preferred embodiments, DNA is ligated into a vector, and introduced into suitable host cells to produce transformed cell lines that express a specific human nNAChR receptor subtype, or specific combinations of subtypes. The resulting cell lines can then be produced in quantity for reproducible quantitative analysis of the effects of drugs on receptor function. In other embodiments, mRNA may be produced by *in vitro*

transcription of DNA encoding each subunit. This mRNA, either from a single subunit clone or from a combination of clones, can then be injected into *Xenopus* oocytes where the mRNA directs the synthesis of the human receptor subunits, which then form functional receptors. Alternatively, the subunit-encoding DNA can be directly injected into oocytes for expression of functional receptors. The transfected mammalian cells or injected oocytes may then be used in the methods of drug screening provided herein.

Cloned full-length DNA encoding any human neuronal nicotinic AChR subunit(s) may be introduced into a plasmid vector for expression in a eukaryotic cell. Such DNA may be genomic DNA or cDNA. Host cells may be transfected with one or a combination of plasmids, each of which encodes at least one human neuronal nicotinic AChR subunit.

Eukaryotic cells in which DNA or RNA may be introduced include any cells that are transfectable by such DNA or RNA or into which such DNA or RNA may be injected. Preferred cells are those that can be transiently or stably transfected and also express the DNA and RNA. Presently most preferred cells are those that can form recombinant or heterologous human neuronal nicotinic AChRs comprising one or more subunits encoded by the heterologous DNA. Such cells may be identified empirically or selected from among those known to be readily transfected or injected.

Exemplary cells for introducing DNA include cells of mammalian origin (e.g., COS cells, mouse L cells, Chinese hamster ovary (CHO) cells, human embryonic kidney cells, African green monkey cells and other such cells known to those of skill in the art), amphibian cells (e.g., *Xenopus laevis* oöcytes), yeast cells (e.g.,

Saccharomyces cerevisiae, *Pichia pastoris*), and the like. Exemplary cells for expressing injected RNA transcripts include *Xenopus laevis* oöcytes. Cells that are preferred for transfection of DNA are known to those of skill in the art or may be empirically identified, and include HEK 293 (which are available from ATCC under accession #CRL 1573); Ltk$^-$ cells (which are available from ATCC under accession #CCL1.3); COS-7 cells (which are available from ATCC under accession #CRL 1651); and DG44 cells (dhfr$^-$ CHO cells; see, e.g., Urlaub et al. (1986) Cell. Molec. Genet. 12: 555). Presently preferred cells include DG44 cells and HEK 293 cells, particularly HEK 293 cells that have been adapted for growth in suspension and that can be frozen in liquid nitrogen and then thawed and regrown. HEK 293 cells are described, for example, in U.S. Patent No. 5,024,939 to Gorman (see, also, Stillman et al. (1985) Mol. Cell. Biol. 5:2051-2060).

DNA may be stably incorporated into cells or may be transiently introduced using methods known in the art. Stably transfected mammalian cells may be prepared by transfecting cells with an expression vector having a selectable marker gene (such as, for example, the gene for thymidine kinase, dihydrofolate reductase, neomycin resistance, and the like), and growing the transfected cells under conditions selective for cells expressing the marker gene. To produce such cells, the cells should be transfected with a sufficient concentration of subunit-encoding nucleic acids to form human neuronal nicotinic AChRs that contain the human subunits encoded by heterologous DNA. The precise amounts and ratios of DNA encoding the subunits may be empirically determined and optimized for a particular combination of subunits, cells and assay conditions. Recombinant cells that express neuronal nicotinic AChR containing subunits encoded only by the heterologous DNA or RNA are especially preferred.

Heterologous DNA may be maintained in the cell as an episomal element or may be integrated into chromosomal DNA of the cell. The resulting recombinant cells may then be cultured or subcultured (or passaged, in the case of mammalian cells) from such a culture or a subculture thereof. Methods for transfection, injection and culturing recombinant cells are known to the skilled artisan. Similarly, the human neuronal nicotinic AChR subunits may be purified using protein purification methods known to those of skill in the art. For example, antibodies or other ligands that specifically bind to one or more of the subunits may be used for affinity purification of the subunit or human neuronal nicotinic AChRs containing the subunits.

In accordance with one embodiment of the present invention, methods for producing cells that express human neuronal nicotinic AChR subunits and functional receptors are also provided. In one such method, host cells are transfected with DNA encoding an α_2 subunit of a neuronal nicotinic acetylcholine receptor and a beta subunit of a neuronal nicotinic acetylcholine receptor. Using methods such as northern blot or slot blot analysis, transfected cells that contain alpha α_2 and, optionally, beta subunit encoding DNA or RNA, can be selected. Transfected cells are also analyzed to identify those that express NAChR protein. Analysis can be carried out, for example, by measuring the ability of cells to bind acetylcholine, nicotine, or a nicotine agonist, compared to the nicotine binding ability of untransfected host cells or other suitable control cells, by electrophysiologically monitoring the currents through the cell membrane in response to a nicotine agonist, and the like.

As used herein, a human beta subunit gene is a gene that encodes a beta subunit of a human neuronal

nicotinic acetylcholine receptor. Assignment of the name "beta" to a putative nNAChR subunit, according to Deneris et al. supra, is based on the lack of adjacent cysteine residues (which are characteristic of alpha subunits). The beta subunit is frequently referred to as the structural NAChR subunit (although it is possible that beta subunits also have ACh binding properties). Combination of beta subunit(s) with appropriate alpha subunit(s) leads to the formation of a functional receptor. A beta subunit forms a functional NAChR, as assessed by methods described herein or known to those of skill in this art, with appropriate alpha subunit subtype(s).

In particularly preferred aspects, eukaryotic cells which contain heterologous DNAs express such DNA and form recombinant functional neuronal nicotinic AChR(s). In more preferred aspects, recombinant neuronal nicotinic AChR activity is readily detectable because it is a type that is absent from the untransfected host cell or is of a magnitude not exhibited in the untransfected cell. Such cells that contain recombinant receptors could be prepared, for example, by causing cells transformed with DNA encoding human neuronal nicotinic AChR α_2 and β_2 subunits to express the corresponding proteins. The resulting synthetic or recombinant receptor would contain only the α_2 and β_2 nNAChR subunits. Such a receptor would be useful for a variety of applications, e.g., as part of an assay system free of the interferences frequently present in prior art assay systems employing non-human receptors or human tissue preparations. Furthermore, testing of single receptor subunits with a variety of potential agonists or antagonists would provide additional information with respect to the function and activity of the individual subunits. Such information is expected to lead to the identification of compounds which are capable of very

specific interaction with one or more of the receptor subunits. Such specificity may prove of great value in medical application.

Thus, DNA encoding one or more human neuronal nicotinic AChR subunits may be introduced into suitable host cells (e.g., eukaryotic or prokaryotic cells) for expression of individual subunits and functional NAChRs. Preferably combinations of alpha and beta subunits may be introduced into cells: such combinations include combinations of α_2, optionally in the further presence of any one or more of α_1, α_3, α_4, α_5 and α_7, with β_2 or β_4.

Sequence information for α_1 is presented in Biochem. Soc. Trans. (1989) 17:219-220; sequence information for α_2 is presented herein; sequence information for α_3 is presented in United States Serial No. 07/504,455, filed April 3, 1990, now pending; sequence information for α_4 is presented in United States Serial No. 08/028,031, filed March 8, 1993, now pending; sequence information for α_5 is presented in Proc. Natl. Acad. Sci.USA (1992) 89:1572-1576; and sequence information for α_7 is presented in United States Serial No. 08/028,031, filed March 8, 1993, now pending.

Sequence information for β_2 is presented in United States Serial No. 07/504,455, filed April 3, 1990, now pending; and sequence information for β_4 is presented in United States Serial No. 08/028,031, filed March 8, 1993, now pending.

In certain embodiments, eukaryotic cells with heterologous human neuronal nicotinic AChRs are produced by introducing into the cell a first composition, which contains at least one RNA transcript that is translated in the cell into a subunit of a human neuronal nicotinic AChR. In preferred embodiments, the composition that is

introduced contains an RNA transcript which encodes a human α_2 subunit and also contains an RNA transcript which encodes a beta subunit of a human neuronal nicotinic AChR. RNA transcripts can be obtained from cells transfected with DNAs encoding human neuronal nicotinic acetylcholine receptor subunits or by *in vitro* transcription of subunit-encoding DNAs. Methods for *in vitro* transcription of cloned DNA and injection of the resulting mRNA into eukaryotic cells are well known in the art. Amphibian oocytes are particularly preferred for expression of *in vitro* transcripts of the human nNAChR DNA clones provided herein. See, for example, Dascal (1989) CRC Crit. Rev. Biochem. 22:317-387, for a review of the use of *Xenopus* oocytes to study ion channels.

Thus, pairwise (or stepwise) introduction of DNA or RNA encoding alpha and beta subtypes into cells is possible. The resulting cells may be tested by the methods provided herein or known to those of skill in the art to detect functional AChR activity. Such testing will allow the identification of pairs of alpha and beta subunit subtypes that produce functional AChRs, as well as individual subunits that produce functional AChRs.

As used herein, activity of a human neuronal nicotinic AChR refers to any activity characteristic of an NAChR. Such activity can typically be measured by one or more *in vitro* methods, and frequently corresponds to an *in vivo* activity of a human neuronal nicotinic AChR. Such activity may be measured by any method known to those of skill in the art, such as, for example, measuring the amount of current which flows through the recombinant channel in response to a stimulus.

Methods to determine the presence and/or activity of human neuronal nicotinic AChRs include assays

that measure nicotine binding, ^{86}Rb ion-flux, Ca^{2+} influx, the electrophysiological response of cells, the electrophysiological response of oocytes transfected with RNA from the cells, and the like. In particular, methods are provided herein for the measurement or detection of an AChR-mediated response upon contact of cells containing the DNA or mRNA with a test compound.

As used herein, a recombinant or heterologous human neuronal nicotinic AChR refers to a receptor that contains one or more subunits encoded by heterologous DNA that has been introduced into and expressed in cells capable of expressing receptor protein. A recombinant human neuronal nicotinic AChR may also include subunits that are produced by DNA endogenous to the host cell. In certain embodiments, recombinant or heterologous human neuronal nicotinic AChR may contain only subunits that are encoded by heterologous DNA.

As used herein, heterologous or foreign DNA and RNA are used interchangeably and refer to DNA or RNA that does not occur naturally as part of the genome of the cell in which it is present or to DNA or RNA which is found in a location or locations in the genome that differ from that in which it occurs in nature. Typically, heterologous or foreign DNA and RNA refers to DNA or RNA that is not endogenous to the host cell and has been artificially introduced into the cell. Examples of heterologous DNA include DNA that encodes a human neuronal nicotinic AChR subunit, DNA that encodes RNA or proteins that mediate or alter expression of endogenous DNA by affecting transcription, translation, or other regulatable biochemical processes, and the like. The cell that expresses heterologous DNA may contain DNA encoding the same or different expression products. Heterologous DNA need not be expressed and may be

integrated into the host cell genome or maintained episomally.

Recombinant receptors on recombinant eukaryotic cell surfaces may contain one or more subunits encoded by the DNA or mRNA encoding human neuronal nicotinic AChR subunits, or may contain a mixture of subunits encoded by the host cell and subunits encoded by heterologous DNA or mRNA. Recombinant receptors may be homogeneous or may be a mixture of subtypes. Mixtures of DNA or mRNA encoding receptors from various species, such as rats and humans, may also be introduced into the cells. Thus, a cell can be prepared that expresses recombinant receptors containing only α_2 and either β_2 or β_4 subunits.

As used herein, a functional neuronal nicotinic AChR is a receptor that exhibits an activity of neuronal nicotinic AChRs as assessed by any *in vitro* or *in vivo* assay disclosed herein or known to those of skill in the art. Possession of any such activity that may be assessed by any method known to those of skill in the art and provided herein is sufficient to designate a receptor as functional. Methods for detecting NAChR protein and/or activity include, for example, assays that measure nicotine binding, ^{86}Rb ion-flux, Ca^{2+} influx, the electrophysiological response of cells containing heterologous DNA or mRNA encoding one or more receptor subunit subtypes, and the like. As used herein, "functional" with respect to a recombinant or heterologous human neuronal nicotinic AChR means that the receptor channel is able to provide for and regulate entry of human neuronal nicotinic AChR-permeable ions, such as, for example, Na^+, K^+, Ca^{2+} or Ba^{2+}, in response to a stimulus and/or bind ligands with affinity for the receptor. Preferably such human neuronal nicotinic AChR activity is distinguishable, such as by electrophysiological, pharmacological and other means

known to those of skill in the art, from any endogenous nicotinic AChR activity that may be produced by the host cell.

In accordance with a particular embodiment of the present invention, recombinant human neuronal nicotinic AChR-expressing mammalian cells or oocytes can be contacted with a test compound, and the modulating effect(s) thereof can then be evaluated by comparing the AChR-mediated response in the presence and absence of test compound, or by comparing the AChR-mediated response of test cells, or control cells (i.e., cells that do not express nNAChRs), to the presence of the compound.

As used herein, a compound or signal that "modulates the activity of a neuronal nicotinic AChR" refers to a compound or signal that alters the activity of NAChR so that activity of the NAChR is different in the presence of the compound or signal than in the absence of the compound or signal. In particular, such compounds or signals include agonists and antagonists. The term agonist refers to a substance or signal, such as ACh, that activates receptor function; and the term antagonist refers to a substance that interferes with receptor function. Typically, the effect of an antagonist is observed as a blocking of activation by an agonist. Antagonists include competitive and non-competitive antagonists. A competitive antagonist (or competitive blocker) interacts with or near the site specific for the agonist (e.g., ligand or neurotransmitter) for the same or closely situated site. A non-competitive antagonist or blocker inactivates the functioning of the receptor by interacting with a site other than the site that interacts with the agonist.

As understood by those of skill in the art, assay methods for identifying compounds that modulate

human neuronal nicotinic AChR activity (e.g., agonists and antagonists) generally require comparison to a control. One type of a "control" cell or "control" culture is a cell or culture that is treated substantially the same as the cell or culture exposed to the test compound, except the control culture is not exposed to test compound. For example, in methods that use voltage clamp electrophysiological procedures, the same cell can be tested in the presence and absence of test compound, by merely changing the external solution bathing the cell. Another type of "control" cell or "control" culture may be a cell or a culture of cells which are identical to the transfected cells, except the cells employed for the control culture do not express functional human neuronal nicotinic AChRs. In this situation, the response of test cell to test compound is compared to the response (or lack of response) of receptor-negative (control) cell to test compound, when cells or cultures of each type of cell are exposed to substantially the same reaction conditions in the presence of compound being assayed.

Functional recombinant human neuronal nicotinic AChRs contemplated by the present invention include at least an α_2 subunit, or an α_2 subunit and a beta subunit of a human neuronal nicotinic AChR. Eukaryotic cells expressing these subunits have been prepared by injection of RNA transcripts and by transfection of DNA. Such cells have exhibited nicotinic AChR activity attributable to human neuronal nicotinic AChRs that contain one or more of the heterologous human neuronal nicotinic AChR subunits.

With respect to measurement of the activity of functional heterologous human neuronal nicotinic AChRs, endogenous AChR activity and, if desired, activity of AChRs that contain a mixture of endogenous host cell

subunits and heterologous subunits, should, if possible, be inhibited to a significant extent by chemical, pharmacological and electrophysiological means.

The invention will now be described in greater detail with reference to the following non-limiting examples.

Example 1

Isolation of DNA Encoding Human nNAChR α_2 Subunits

RNA isolated from human thalamus tissue was used in synthesizing cDNA for λgt11-based cDNA library. The library constructed from the cDNAs was screened for hybridization to a fragment of a rat nicotinic AChR α_2 subunit cDNA, using low stringency wash conditions.

Hybridizing clones were plaque-purified and characterized by restriction enzyme mapping and DNA sequence analysis. The insert DNA of one of the clones contained a translation initiation codon and nearly the complete coding sequence of an α_2 subunit of a human nicotinic AChR, except for ~77 bp at the 3' end. This insert was ligated with the insert of another clone containing the complete 3' end of the α_2 subunit coding sequence to generate a full-length α_2 subunit cDNA. The full-length cDNA was isolated and ligated into the polylinker of vector pIBI24 to generate hα2/pIBI24.

To verify the α2 subunit coding sequence in hα2/pIBI24 near nucleotide 450, human thalamus cDNAs were subjected to nucleic acid amplification using oligonucleotides SE153 and SE154 as primers. These oligonucleotides correspond to human α2 subunit-coding sequence located 5' and 3' of nucleotide 450. SE153, corresponding to the extreme 5' end of the α2 subunit-coding sequence, also contained nucleotides corresponding

to a consensus ribosome binding site (RBS). Consequently, the product of the amplification contained an RBS (GCCACC) immediately 5' of the translation initiation codon.

The expected ~660-bp product was obtained from the amplification reaction. The product was digested with *Eco*RI/*Sst*I and an ~560-bp fragment, comprising the 5' end of the α2 subunit coding sequence, was ligated with *Eco*RI/*Sst*I-digested pIBI24 to create hα2-5'/PIBI. To generate full-length α2 subunit cDNA, a DNA fragment containing the 3' end of the α2 subunit coding sequence was ligated to the 5' fragment contained in hα2-5'/PIBI as follows.

Plasmid hα2/PIBI24 was digested with *Cla*I, followed by partial digestion with *Sst*I. The 1.7-kb fragment containing the 3' portion of the α2 subunit coding sequence was isolated and ligated with SstI-digested hα2-5'/PIBI to create PIBI-KEα2RBS. This construct contains the correct full-length α2 subunit coding sequence preceded immediately by an RBS.

Example 2

Preparation of Constructs for the Expression of Recombinant Human Neuronal Nicotinic AChR α_2 Subunits

Isolated cDNAs encoding human neuronal nicotinic AChR subunits were incorporated into vectors for use in expressing the subunits in mammalian host cells and for use in generating *in vitro* transcripts of the DNAs to be expressed in *Xenopus* oocytes. Several different vectors were utilized in preparing the constructs as follows.

A. Construct for Expression of a Human nNAChR α_2 Subunit

DNA encoding a human neuronal nicotinic AChR α_2 subunit was subcloned into the pCMV-T7-3 general expression vector to create pCMV-KEα2RBSf. Plasmid pCMV-T7-3 (see Figure 1) is a pUC19-based vector that contains a CMV promoter/enhancer, SV40 splice donor/splice acceptor sites located immediately downstream of the promoter, a T7 bacteriophage RNA polymerase promoter positioned downstream of the SV40 splice sites, an SV40 polyadenylation signal downstream of the T7 promoter, and a polylinker between the T7 promoter and the polyadenylation signal. This vector thus contains all the regulatory elements required for expression of heterologous DNA in a mammalian host cell, wherein the heterologous DNA has been incorporated into the vector at the polylinker. In addition, because the T7 promoter is located just upstream of the polylinker, this plasmid can be used for synthesis of *in vitro* transcripts of heterologous DNA that has been subcloned into the vector at the polylinker. Figure 1 also shows a restriction map of pCMV-T7-2. This plasmid is identical to pCMV-T7-3 except that the restriction sites in the polylinker are in the opposite order as compared to the order in which they occur in pCMV-T7-3.

The insert in pIBI-KEα2RBS (see Example 1) was isolated as an *Eco*RI fragment and ligated with *Eco*RI-digested pCMV-T7-3 to generate pCMV-KEα2RBSf. Construct pCMV-KEα2RBSf contains the α2 subunit coding sequence preceded immediately by an RBS and followed by 316 bp of 3' untranslated sequence. The coding sequence is operably positioned downstream of the T7 promoter thereby enabling the generation of *in vitro* transcripts of the cDNA.

B. Construct for Expression of a Human nNAChR β_2 Subunit

DNA encoding a human NAChR ß2 subunit (see U.S. Patent Application Serial Nos. 07/504,455 and 08/028,031 for such DNAs) was incorporated into expression vector pSP64T. Vector pSP64T [see Krieg and Melton (1984) *Nuc. Acids Res.* 12:7057-7070] is a modified form of vector pSP64 (Promega). The human NAChR ß2 subunit coding sequence (preceded by an RBS) was incorporated into pSP64T at a unique restriction enzyme cloning site that is flanked by 5' and 3' untranslated sequences from the *Xenopus* ß-globin gene. These sequences are located downstream of the SP6 promoter contained in pSP64T. The resulting vector, pSP64T-KEß2RBS1, contains the human NAChR ß2 subunit coding sequence in operable association with SP6 transcription regulatory regions for the production of *in vitro* transcripts of the heterologous DNA.

Example 3

Expression of Recombinant Human Nicotinic AChR in Oocytes

Xenopus oocytes were injected with *in vitro* transcripts prepared from constructs containing DNA encoding α_2 and β_2 subunits. Electrophysiological measurements of the oocyte transmembrane currents were made using the two-electrode voltage clamp technique (see, e.g., Stuhmer (1992) *Meth. Enzymol. 207*:319-339).

1. Preparation of *in vitro* transcripts

Recombinant capped transcripts of pCMV-KEα2RBSf were synthesized from linearized plasmids using the mMessage mMachine T7 (Ambion Cat. #1334). Recombinant capped transcripts of pSP64T-KEß2RBS1 were synthesized from linearized plasmids using the MEGAscript SP6 *in*

vitro transcription kit according to the capped transcript protocol provided by the manufacturer (Catalog #1330 from AMBION, Inc., Austin, TX). The mass of each synthesized transcript was determined by UV absorbance and the integrity of each transcript was determined by electrophoresis through an agarose gel.

2. Electrophysiology

Xenopus oocytes were injected with 10 ng of each human nicotinic AChR subunit transcript per oocyte. The preparation and injection of oocytes were carried out as described by Dascal (1987) in *Crit. Rev. Biochem.* *22*:317-387. Two-to-six days following mRNA injection, the oocytes were examined using the two-electrode voltage clamp technique. The cells were bathed in Ringer's solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 10 mM HEPES, pH 7.3) containing 1 μM atropine. Cells were voltage-clamped at -80 mV. Data were acquired with Axotape software (AXON Instruments) at ~2 Hz. The agonists acetylcholine (ACh), nicotine, 1,1-dimethyl-4-phenylpiperazinium (DMPP) and cytisine were added at a concentration of about 1 μM.

For the purpose of comparing the relative potencies of the four different agonists (i.e., nicotine, acetylcholine, cytisine and DMPP), oocytes were exposed to 1 μM doses of each agonist, and the current responses recorded. The responses were normalized to the response of 1 μM ACh (designated as 1.00 (see following table).

	$\alpha_2\beta_2$ Normalized Response
ACh	1.00
Nicotine	0.33 ± 0.06
DMPP	0.40 ± 0.15
Cytosine	0.09 ± 0.04

n = 4

The recombinant oocytes exhibited detectable current responses to all four agonists. The data in the table presented above demonstrate that NAChRs expressed in oocytes injected with transcripts of human α_2 and β_2 subunit cDNAs have the following relative sensitivities to the agonists:

ACh > DMPP ≈ nicotine > cytisine

Example 4

Recombinant Expression of Human nNAChR α_2 Subunits in Mammalian Cells

Human embryonic kidney (HEK) 293 cells or other suitable host cells can be transiently and stably transfected with DNA encoding human neuronal nicotinic AChR alpha and beta subunits. Transfectants are analyzed for expression of nicotinic AChR using various assays, e.g., electrophysiological methods, Ca^{2+}-sensitive fluorescent indicator-based assays and nicotine-binding assays.

A. Transient Transfection of Host Cells

In transient transfections, ~2 x 10^6 host cells, e.g., HEK293 cells, are transiently transfected with 18 μg of plasmid(s) containing NAChR subunit DNAs according to standard $CaPO_4$ transfection procedures [Wigler *et al.*

(1979) *Proc. Natl. Acad. Sci. USA* 76:1373-1376]. In addition, 2 μg of plasmid pCMVβgal (Clontech Laboratories, Palo Alto, CA), which contains the *Escherichia coli* β-galactosidase gene fused to the CMV promoter, are co-transfected as a reporter gene for monitoring the efficiency of transfection. The transfectants are analyzed for β-galactosidase expression by measurement of β-galactosidase activity [Miller (1972) Experiments in Molecular Genetics, pp.352-355, Cold Spring Harbor Press]. Transfectants can also be analyzed for β-galactosidase expression by direct staining of the product of a reaction involving β-galactosidase and the X-gal substrate [Jones (1986) *EMBO* 5:3133-3142].

B. Stable Transfection of Host Cells

For stable transfection, host cells, such as HEK 293 cells, can be transfected using the calcium phosphate transfection procedure [*Current Protocols in Molecular Biology, Vol. 1*, Wiley Inter-Science, Supplement 14, Unit 9.1.1-9.1.9 (1990)]. Ten-cm plates, each containing one-to-two million host cells are transfected with 1 ml of DNA/calcium phosphate precipitate containing ~19 μg total of NAChR α and β subunit construct DNA, and 1 μg selectable marker DNA, e.g., pSV2neo. Typically, after 14 days of growth in selection media containing, for example, 1 μg/ml G418, colonies form and are individually isolated by using cloning cylinders. The isolates are subjected to limiting dilution and screened to identify those that express the highest level of nicotinic AChR, as described below.

Example 5

Characterization of Cell Lines Expressing nNAChRs

Recombinant cell lines generated by transfection with DNA encoding human neuronal nicotinic AChRs, such as those described in Example 3, can be further characterized using one or more of the following methods.

A. Fluorescent indicator-based assays

Activation of the ligand-gated nicotinic AChR by agonists leads to an influx of cations, including Ca^{++}, through the receptor channel. Ca^{++} entry into the cell through the channel can induce release of calcium contained in intracellular stores. Monovalent cation entry into the cell through the channel can also result in an increase in cytoplasmic Ca^{++} levels through depolarization of the membrane and subsequent activation of voltage-dependent calcium channels. Therefore, methods of detecting transient increases in intracellular calcium concentration can be applied to the analysis of functional nicotinic AChR expression. One method for measuring intracellular calcium levels relies on calcium-sensitive fluorescent indicators.

Calcium-sensitive indicators, such as fluo-3 (Catalog No. F-1241, Molecular Probes, Inc., Eugene, OR), are available as acetoxymethyl esters which are membrane permeable. When the acetoxymethyl ester form of the indicator enters a cell, the ester group is removed by cytosolic esterases, thereby trapping the free indicator in the cytosol. Interaction of the free indicator with calcium results in increased fluorescence of the indicator; therefore, an increase in the intracellular Ca^{2+} concentration of cells containing the indicator can be expressed directly as an increase in fluorescence. An

automated fluorescence detection system for assaying nicotinic AChR has been described in commonly assigned pending US Patent Application No. 07/812,254 and corresponding PCT Patent Application No. US92/11090.

Host cells that are transiently or stably co-transfected with DNA encoding alpha and beta subunits can be analyzed for expression of functional recombinant nicotinic AChR using the automated fluorescent indicator-based assay. The assay procedure is as follows.

Untransfected host cells and host cells that have been transfected with DNA encoding alpha and beta subunits are plated in the wells of a 96-well microtiter dish and loaded with fluo-3 by incubation for 2 hours at 20°C in a medium containing 20 μM fluo-3, 0.2% Pluronic F-127 in HBS (125 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.62 mM $MgSO_4$, 6 mM glucose, 20 mM HEPES, pH 7.4). The cells are then washed with assay buffer (i.e., HBS). The antagonist d-tubocurarine can be added to some of the wells at a final concentration of 10 μM. The microtiter dish is then placed into a fluorescence plate reader and the basal fluorescence of each well is measured and recorded before addition of 200 μM nicotine to the wells. The fluorescence of the wells is monitored repeatedly during a period of approximately 60 seconds following addition of nicotine.

B. Northern or slot blot analysis for expression of α- and/or β-subunit encoding messages

Total RNA is isolated from ~$1X10^7$ cells and 10-15 μg of RNA from each cell type is used for northern or slot blot hybridization analysis. The inserts from human neuronal NAChR-encoding plasmids can be nick-translated and used as probe. In addition, the β-actin gene sequence (Cleveland et al. (1980) Cell 20:95-105) can b nick-translated and used as a control probe on

duplicate filters to confirm the presence or absence of RNA on each blot and to provide a rough standard for use in quantitating differences in α- or β-specific mRNA levels between cell lines. Typical northern and slot blot hybridization and wash conditions are as follows:

hybridization in 5x SSPE, 5X Denhardt's solution, 50% formamide, at 42°C followed by washing in 0.2x SSPE, 0.1% SDS, at 65°C.

C. Nicotine-binding assay

Cell lines generated by transfection with human neuronal nicotinic AChR α- or α- and β-subunit-encoding DNA can be analyzed for their ability to bind nicotine, for example, as compared to control cell lines: neuronally-derived cell lines PC12 (Boulter et al., (1986), supra; ATCC #CRL1721) and IMR32 (Clementi, et al. (1986); Int. J. Neurochem. 47:291-297; ATCC #CCL127), and muscle-derived cell line BC3H1 (Patrick, et al., (1977); J. Biol. Chem. 252:2143-2153). Negative control cells (i.e., host cells from which the transfectants were prepared) are also included in the assay. The assay is conducted as follows:

Just prior to being assayed, transfected cells are removed from plates by scraping. Positive control cells used are PC12, BC3H1, and IMR32 (which had been starved for fresh media for seven days). Control cell lines are removed by rinsing in 37°C assay buffer (50mM Tris/HCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 120 mM NaCl, 3 mM EDTA, 2 mg/ml BSA and 0.1 % aprotinin at pH7.4). The cells are washed and resuspended to a concentration of 1 x 10^6/250 µl. To each plastic assay tube is added 250 µl of the cell solution, 15 nM ^{3}H-nicotine, with or without 1 mM unlabeled nicotine, and assay buffer to make a final volume of 500 µl. The assays for the transfected cell

lines are incubated for 30 min at room temperature; the assays of the positive control cells are incubated for 2 min at 1°C. After the appropriate incubation time, 450 µl aliquots of assay volume are filtered through Whatman GF/C glass fiber filters which has been pretreated by incubation in 0.05% polyethyleneimine for 24 hours at 4°C. The filters are then washed twice, with 4 ml each wash, with ice cold assay buffer. After washing, the filters are dried, added to vials containing 5 ml scintillation fluid and radioactivity is measured.

D. ^{86}Rb ion-flux assay

The ability of nicotine or nicotine agonists and antagonists to mediate the influx of ^{86}Rb into transfected and control cells has been found to provide an indication of the presence of functional AChRs on the cell surface. The ^{86}Rb ion-flux assay is conducted as follows:

1. The night before the experiment, cells are plated at 2 x 10^6 per well (i.e., 2 ml per well) in a 6-well polylysine-coated plate.

2. The culture medium is decanted and the plate washed with 2 ml of assay buffer (50 mM HEPES, 260 mM sucrose, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5.5. mM glucose) at room temperature.

3. The assay buffer is decanted and 1 ml of assay buffer, containing 3 µCi/ml ^{86}Rb, with 5 mM ouabain and agonist or antagonist in a concentration to effect a maximum response, is added.

4. The plate is incubated on ice at 1°C for 4 min.

5. The buffer is decanted into a waste container and each well was washed with 3 ml of assay buffer, followed by two washes of 2 ml each.

6. The cells are lysed with 2 x 0.5 ml of 0.2% SDS per well and transferred to a scintillation vial containing 5 ml of scintillation fluid.

7. The radioactivity contained in each vial is measured and the data calculated.

Positive control cells provided the following data in this assay:

	PC12		IMR32	
	EC_{50}	Maximum response	EC_{50}	Maximum response
Agonist				
nicotine	52 μM	2.1X[c]	18 μM	7.7X[a]
CCh*	35 μM	3.3X[b]	230 μM	7.6X[c]
cytisine	57 μM	3.6X[d]	14 μM	10X[e]
Antagonist				
d-tubocurarine	0.81 μM		2.5 μM	
mecamylamine	0.42 μM		0.11 μM	
hexamethonium	nd[f]		22 μM	
atropine	12.5 μM		43 μM	

* CCh = carbamylcholine
[a] 200μM nicotine
[b] 300μM CCh
[c] 3mM CCh
[d] 1mM cytisine
[e] 100μM cytisine
[f] nd = not determined

E. Electrophysiological Analysis of Mammalian Cells Transfected with Human Neuronal Nicotinic AChR Subunit-encoding DNA

Electrophysiological measurements may be used to assess the activity of recombinant receptors or to assess the ability of a test compound to potentiate, antagonize or otherwise modulate the magnitude and duration of the flow of cations through the ligand-gated recombinant AChR. The function of the expressed neuronal AChR can be assessed by a variety of electrophysiological techniques, including two-electrode voltage clamp and patch clamp methods. The cation-conducting channel intrinsic to the AChR opens in response to acetylcholine (ACh) or other nicotinic cholinergic agonists, permitting

the flow of transmembrane current carried predominantly by sodium and potassium ions under physiological conditions. This current can be monitored directly by voltage clamp techniques. In preferred embodiments, transfected mammalian cells or injected oocytes are analyzed electrophysiologically for the presence of AChR agonist-dependent currents.

While the invention has been described in detail with reference to certain preferred embodiments thereof, it will be understood that modifications and variations are within the spirit and scope of that which is described and claimed.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Elliot, Kathryn J.
Ellis, Steven B.
Harpold, Michael M.

(ii) TITLE OF INVENTION: HUMAN NEURONAL NICOTINIC ACETYLCHOLINE RECEPTOR COMPOSITIONS AND METHODS EMPLOYING SAME

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Pretty, Schroeder, Brueggemann & Clark
(B) STREET: 444 South Flower Street, Suite 2000
(C) CITY: Los Angeles
(D) STATE: CA
(E) COUNTRY: USA
(F) ZIP: 90071

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE: 08-NOV-1993
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/028,031
(B) FILING DATE: 08-MAR-1993

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Reiter, Stephen E.
(B) REGISTRATION NUMBER: 31,192
(C) REFERENCE/DOCKET NUMBER: P41 9499

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 619-546-4737
(B) TELEFAX: 619-546-9392

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2277 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 166..1755

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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CCACAGCAAA GCCCTGACCT GACCTCCTGA TGCTCAGGAG AAGCC ATG GGC CCC          174
                                                    Met Gly Pro
                                                      1

TCC TGT CCT GTG TTC CTG TCC TTC ACA AAG CTC AGC CTG TGG TGG CTC        222
Ser Cys Pro Val Phe Leu Ser Phe Thr Lys Leu Ser Leu Trp Trp Leu
  5                  10                  15

CTT CTG ACC CCA GCA GGT GGA GAG GAA GCT AAG CGC CCA CCT CCC AGG        270
Leu Leu Thr Pro Ala Gly Gly Glu Glu Ala Lys Arg Pro Pro Pro Arg
 20                  25                  30                  35

GCT CCT GGA GAC CCA CTC TCC TCT CCC AGT CCC ACG GCA TTG CCG CAG        318
Ala Pro Gly Asp Pro Leu Ser Ser Pro Ser Pro Thr Ala Leu Pro Gln
                 40                  45                  50

GGA GGC TCG CAT ACC GAG ACT GAG GAC CGG CTC TTC AAA CAC CTC TTC        366
Gly Gly Ser His Thr Glu Thr Glu Asp Arg Leu Phe Lys His Leu Phe
             55                  60                  65

CGG GGC TAC AAC CGC TGG GCG CGC CCG GTG CCC AAC ACT TCA GAC GTG        414
Arg Gly Tyr Asn Arg Trp Ala Arg Pro Val Pro Asn Thr Ser Asp Val
         70                  75                  80

GTG ATT GTG CGC TTT GGA CTG TCC ATC GCT CAG CTC ATC GAT GTG GAT        462
Val Ile Val Arg Phe Gly Leu Ser Ile Ala Gln Leu Ile Asp Val Asp
  85                  90                  95

GAG AAG AAC CAA ATG ATG ACC ACC AAC GTC TGG CTA AAA CAG GAG TGG        510
Glu Lys Asn Gln Met Met Thr Thr Asn Val Trp Leu Lys Gln Glu Trp
100                 105                 110                 115

AGC GAC TAC AAA CTG CGC TGG AAC CCC GCT GAT TTT GGC AAC ATC ACA        558
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                120                 125                 130

TCT CTC AGG GTC CCT TCT GAG ATG ATC TGG ATC CCC GAC ATT GTT CTC        606
Ser Leu Arg Val Pro Ser Glu Met Ile Trp Ile Pro Asp Ile Val Leu
            135                 140                 145

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Tyr Asn Asn Ala Asp Gly Glu Phe Ala Val Thr His Met Thr Lys Ala
        150                 155                 160

CAC CTC TTC TCC ACG GGC ACT GTG CAC TGG GTG CCC CCG GCC ATC TAC        702
His Leu Phe Ser Thr Gly Thr Val His Trp Val Pro Pro Ala Ile Tyr
    165                 170                 175

AAG AGC TCC TGC AGC ATC GAC GTC ACC TTC TTC CCC TTC GAC CAG CAG        750
Lys Ser Ser Cys Ser Ile Asp Val Thr Phe Phe Pro Phe Asp Gln Gln
180                 185                 190                 195

AAC TGC AAG ATG AAG TTT GGC TCC TGG ACT TAT GAC AAG GCC AAG ATC        798
Asn Cys Lys Met Lys Phe Gly Ser Trp Thr Tyr Asp Lys Ala Lys Ile
                200                 205                 210

GAC CTG GAG CAG ATG GAG CAG ACT GTG GAC CTG AAG GAC TAC TGG GAG        846
Asp Leu Glu Gln Met Glu Gln Thr Val Asp Leu Lys Asp Tyr Trp Glu
            215                 220                 225

AGC GGC GAG TGG GCC ATC GTC AAT GCC ACG GGC ACC TAC AAC AGC AAG        894
Ser Gly Glu Trp Ala Ile Val Asn Ala Thr Gly Thr Tyr Asn Ser Lys
        230                 235                 240
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AAG TAC GAC TGC TGC GCC GAG ATC TAC CCC GAC GTC ACC TAC GCC TTC          942
Lys Tyr Asp Cys Cys Ala Glu Ile Tyr Pro Asp Val Thr Tyr Ala Phe
    245                 250                 255

GTC ATC CGG CGG CTG CCG CTC TTC TAC ACC ATC AAC CTC ATC ATC CCC          990
Val Ile Arg Arg Leu Pro Leu Phe Tyr Thr Ile Asn Leu Ile Ile Pro
260                 265                 270                 275

TGC CTG CTC ATC TCC TGC CTC ACT GTG CTG GTC TTC TAC CTG CCC TCC         1038
Cys Leu Leu Ile Ser Cys Leu Thr Val Leu Val Phe Tyr Leu Pro Ser
                280                 285                 290

GAC TGC GGC GAG AAG ATC ACG CTG TGC ATT TCG GTG CTG CTG TCA CTC         1086
Asp Cys Gly Glu Lys Ile Thr Leu Cys Ile Ser Val Leu Leu Ser Leu
            295                 300                 305

ACC GTC TTC CTG CTG CTC ATC ACT GAG ATC ATC CCG TCC ACC TCG CTG         1134
Thr Val Phe Leu Leu Leu Ile Thr Glu Ile Ile Pro Ser Thr Ser Leu
        310                 315                 320

GTC ATC CCG CTC ATC GGC GAG TAC CTG CTG TTC ACC ATG ATC TTC GTC         1182
Val Ile Pro Leu Ile Gly Glu Tyr Leu Leu Phe Thr Met Ile Phe Val
    325                 330                 335

ACC CTG TCC ATC GTC ATC ACC GTC TTC GTG CTC AAT GTG CAC CAC CGC         1230
Thr Leu Ser Ile Val Ile Thr Val Phe Val Leu Asn Val His His Arg
340                 345                 350                 355

TCC CCC AGC ACC CAC ACC ATG CCC CAC TGG GTG CGG GGG GCC CTT CTG         1278
Ser Pro Ser Thr His Thr Met Pro His Trp Val Arg Gly Ala Leu Leu
                360                 365                 370

GGC TGT GTG CCC CGG TGG CTT CTG ATG AAC CGG CCC CCA CCA CCC GTG         1326
Gly Cys Val Pro Arg Trp Leu Leu Met Asn Arg Pro Pro Pro Pro Val
            375                 380                 385

GAG CTC TGC CAC CCC CTA CGC CTG AAG CTC AGC CCC TCT TAT CAC TGG         1374
Glu Leu Cys His Pro Leu Arg Leu Lys Leu Ser Pro Ser Tyr His Trp
        390                 395                 400

CTG GAG AGC AAC GTG GAT GCC GAG GAG AGG GAG GTG GTG GTG GAG GAG         1422
Leu Glu Ser Asn Val Asp Ala Glu Glu Arg Glu Val Val Val Glu Glu
    405                 410                 415

GAG GAC AGA TGG GCA TGT GCA GGT CAT GTG GCC CCC TCT GTG GGC ACC         1470
Glu Asp Arg Trp Ala Cys Ala Gly His Val Ala Pro Ser Val Gly Thr
420                 425                 430                 435

CTC TGC AGC CAC GGC CAC CTG CAC TCT GGG GCC TCA GGT CCC AAG GCT         1518
Leu Cys Ser His Gly His Leu His Ser Gly Ala Ser Gly Pro Lys Ala
                440                 445                 450

GAG GCT CTG CTG CAG GAG GGT GAG CTG CTG CTA TCA CCC CAC ATG CAG         1566
Glu Ala Leu Leu Gln Glu Gly Glu Leu Leu Leu Ser Pro His Met Gln
            455                 460                 465

AAG GCA CTG GAA GGT GTG CAC TAC ATT GCC GAC CAC CTG CGG TCT GAG         1614
Lys Ala Leu Glu Gly Val His Tyr Ile Ala Asp His Leu Arg Ser Glu
        470                 475                 480

GAT GCT GAC TCT TCG GTG AAG GAG GAC TGG AAG TAT GTT GCC ATG GTC         1662
Asp Ala Asp Ser Ser Val Lys Glu Asp Trp Lys Tyr Val Ala Met Val
    485                 490                 495

ATC GAC AGG ATC TTC CTC TGG CTG TTT ATC ATC GTC TGC TTC CTG GGG         1710
Ile Asp Arg Ile Phe Leu Trp Leu Phe Ile Ile Val Cys Phe Leu Gly
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AATGTTTAGA TTTGGAGATG AGCCCAAAGT GCCAGGGAGA ACAGCCAGGT GAGGTGGGAG   1882

GTTGGAGAGC CAGGTGAGGT CTCTCTAAGT CAGGCTGGGG TTGAAGTTTG GAGTCTGTCC   1942

GAGTTTGCAG GGTGCTGAGC TGTATGGTCC AGCAGGGGAG TAATAAGGGC TCTTCCGGAA   2002

GGGGAGGAAG CGGGAGGCAG GGCCTGCACC TGATGTGGAG GTACAGGGCA GATCTTCCCT   2062

ACCGGGGAGG GATGGATGGT TGGATACAGG TGGCTGGGCT ATTCCATCCA TCTGGAAGCA   2122

CATTTGAGCC TCCAGGCTTC TCCTTGACGT CATTCCTCTC CTTCCTTGCT CCAAAATGGC   2182

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 529 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Met Gly Pro Ser Cys Pro Val Phe Leu Ser Phe Thr Lys Leu Ser Leu
 1               5                  10                  15

Trp Trp Leu Leu Leu Thr Pro Ala Gly Gly Glu Glu Ala Lys Arg Pro
            20                  25                  30

Pro Pro Arg Ala Pro Gly Asp Pro Leu Ser Ser Pro Ser Pro Thr Ala
        35                  40                  45

Leu Pro Gln Gly Gly Ser His Thr Glu Thr Glu Asp Arg Leu Phe Lys
    50                  55                  60

His Leu Phe Arg Gly Tyr Asn Arg Trp Ala Arg Pro Val Pro Asn Thr
65                  70                  75                  80

Ser Asp Val Val Ile Val Arg Phe Gly Leu Ser Ile Ala Gln Leu Ile
                85                  90                  95

Asp Val Asp Glu Lys Asn Gln Met Met Thr Thr Asn Val Trp Leu Lys
            100                 105                 110

Gln Glu Trp Ser Asp Tyr Lys Leu Arg Trp Asn Pro Ala Asp Phe Gly
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Asn Ile Thr Ser Leu Arg Val Pro Ser Glu Met Ile Trp Ile Pro Asp
    130                 135                 140

Ile Val Leu Tyr Asn Asn Ala Asp Gly Glu Phe Ala Val Thr His Met
145                 150                 155                 160

Thr Lys Ala His Leu Phe Ser Thr Gly Thr Val His Trp Val Pro Pro
                165                 170                 175
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Ala Ile Tyr Lys Ser Ser Cys Ser Ile Asp Val Thr Phe Phe Pro Phe
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Asp Gln Gln Asn Cys Lys Met Lys Phe Gly Ser Trp Thr Tyr Asp Lys
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Ala Lys Ile Asp Leu Glu Gln Met Glu Gln Thr Val Asp Leu Lys Asp
    210                 215                 220

Tyr Trp Glu Ser Gly Glu Trp Ala Ile Val Asn Ala Thr Gly Thr Tyr
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Asn Ser Lys Lys Tyr Asp Cys Cys Ala Glu Ile Tyr Pro Asp Val Thr
                245                 250                 255

Tyr Ala Phe Val Ile Arg Arg Leu Pro Leu Phe Tyr Thr Ile Asn Leu
            260                 265                 270

Ile Ile Pro Cys Leu Leu Ile Ser Cys Leu Thr Val Leu Val Phe Tyr
        275                 280                 285

Leu Pro Ser Asp Cys Gly Glu Lys Ile Thr Leu Cys Ile Ser Val Leu
    290                 295                 300

Leu Ser Leu Thr Val Phe Leu Leu Leu Ile Thr Glu Ile Ile Pro Ser
305                 310                 315                 320

Thr Ser Leu Val Ile Pro Leu Ile Gly Glu Tyr Leu Leu Phe Thr Met
                325                 330                 335

Ile Phe Val Thr Leu Ser Ile Val Ile Thr Val Phe Val Leu Asn Val
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His His Arg Ser Pro Ser Thr His Thr Met Pro His Trp Val Arg Gly
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Ala Leu Leu Gly Cys Val Pro Arg Trp Leu Leu Met Asn Arg Pro Pro
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Pro Pro Val Glu Leu Cys His Pro Leu Arg Leu Lys Leu Ser Pro Ser
385                 390                 395                 400

Tyr His Trp Leu Glu Ser Asn Val Asp Ala Glu Glu Arg Glu Val Val
                405                 410                 415

Val Glu Glu Glu Asp Arg Trp Ala Cys Ala Gly His Val Ala Pro Ser
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Val Gly Thr Leu Cys Ser His Gly His Leu His Ser Gly Ala Ser Gly
        435                 440                 445

Pro Lys Ala Glu Ala Leu Leu Gln Glu Gly Glu Leu Leu Leu Ser Pro
    450                 455                 460

His Met Gln Lys Ala Leu Glu Gly Val His Tyr Ile Ala Asp His Leu
465                 470                 475                 480

Arg Ser Glu Asp Ala Asp Ser Ser Val Lys Glu Asp Trp Lys Tyr Val
                485                 490                 495

Ala Met Val Ile Asp Arg Ile Phe Leu Trp Leu Phe Ile Ile Val Cys
            500                 505                 510

Phe Leu Gly Thr Ile Gly Leu Phe Leu Pro Pro Phe Leu Ala Gly Met
        515                 520                 525

Ile
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THAT WHICH IS CLAIMED:

1. Isolated DNA comprising a sequence of nucleotides encoding an α_2 subunit of a human neuronal nicotinic acetylcholine receptor.

2. DNA according to claim 1 wherein the nucleotides of said DNA encode the amino acid sequence set forth in SEQ. ID No. 2.

3. DNA according to claim 1 wherein the nucleotides of said DNA hybridize to substantially the entire coding sequence (nucleotides 166-1755) set forth in SEQ. ID No. 1 under high stringency conditions.

4. DNA according to claim 1 wherein said DNA has substantially the same nucleotide sequence as nucleotides 166-1755 set forth in SEQ. ID No. 1.

5. Cells containing at least one DNA according to claim 1.

6. Cells according to claim 5, wherein said cells are selected from bacterial cells, eukaryotic cells and amphibian oöcytes.

7. Cells according to claim 5, further containing DNA encoding a β subunit of human neuronal nicotinic acetylcholine receptor.

8. Cells according to claim 7 wherein said β subunit is selected from β_2 or β_4.

9. Cells according to claim 5 wherein said cells express functional neuronal nicotinic acetylcholine receptors that contain one or more subunits encoded by said DNA.

10. A method of screening compounds to identify compounds which modulate the activity of human neuronal nicotinic acetylcholine receptors, said method comprising determining the effect of a compound on the neuronal nicotinic acetylcholine receptor activity in test cells according to claim 7, compared to the effect on control cells or to the neuronal nicotinic acetylcholine receptor activity of the cells in the absence of the compound,

wherein control cells are substantially identical to the test cells, but control cells do not express nicotinic acetylcholine receptors.

11. Recombinant human neuronal nicotinic acetylcholine receptor α_2 subunit.

12. A recombinant human neuronal nicotinic acetylcholine receptor comprising the subunit of claim 11.

13. Human neuronal nicotinic acetylcholine receptor according to claim 12, further comprising a human neuronal nicotinic acetylcholine receptor beta subunit.

14. A method for identifying functional neuronal nicotinic acetylcholine receptor subunits and combinations thereof, said method comprising:

(a) introducing at least one DNA according to claim 1, or RNA complementary thereto, and optionally DNA encoding at least one beta subunit of a human neuronal

nicotinic acetylcholine receptor, or RNA complementary thereto, into eukaryotic cells; and

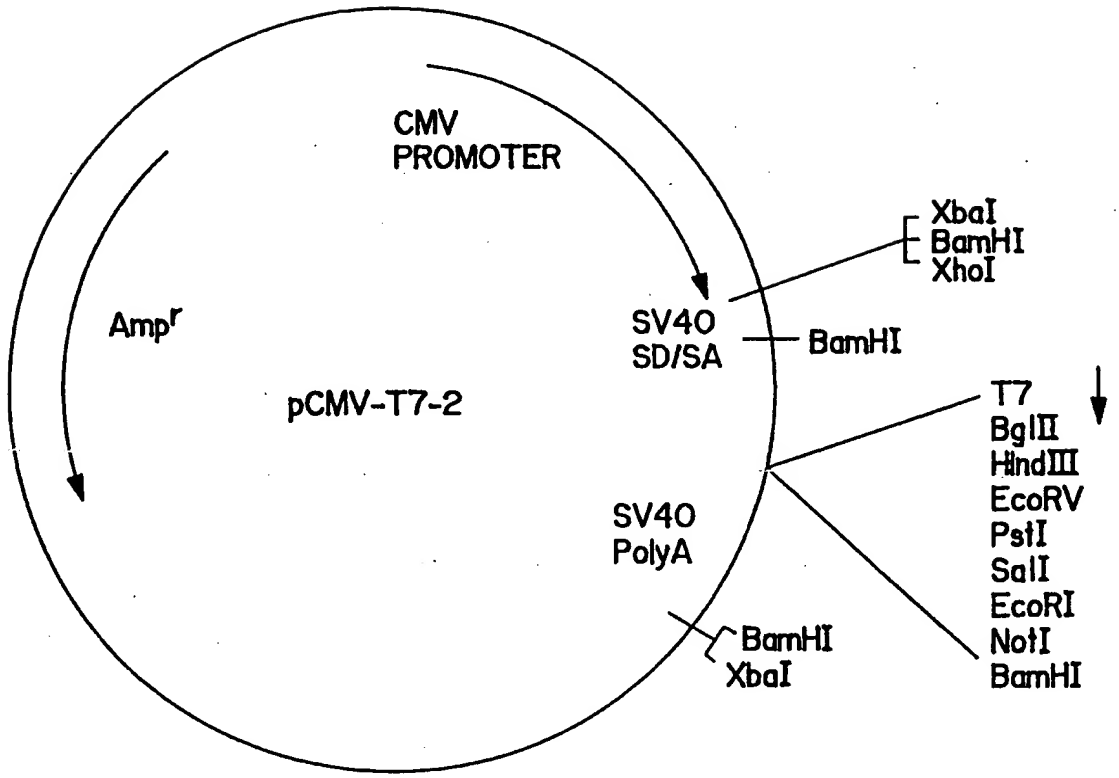
(b) assaying for neuronal nicotinic acetylcholine receptor activity in cells of step (a), wherein the activity is mediated by a receptor containing one or more of the subunits encoded by said introduced DNA.

15. Isolated mRNA encoded by the DNA of Claim 1.

16. Cells containing mRNA according to claim 15.

17. Cells according to claim 16 wherein said cells further contain mRNA encoding a beta subunit of a human neuronal nicotinic acetylcholine receptor.

FIGURE 1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Amp^r
pCMV-T7-2
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SD/SA
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PolyA
XbaI
BamHI
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BglII
HindIII
EcoRV
PstI
SalI
EcoRI
NotI
BamHI
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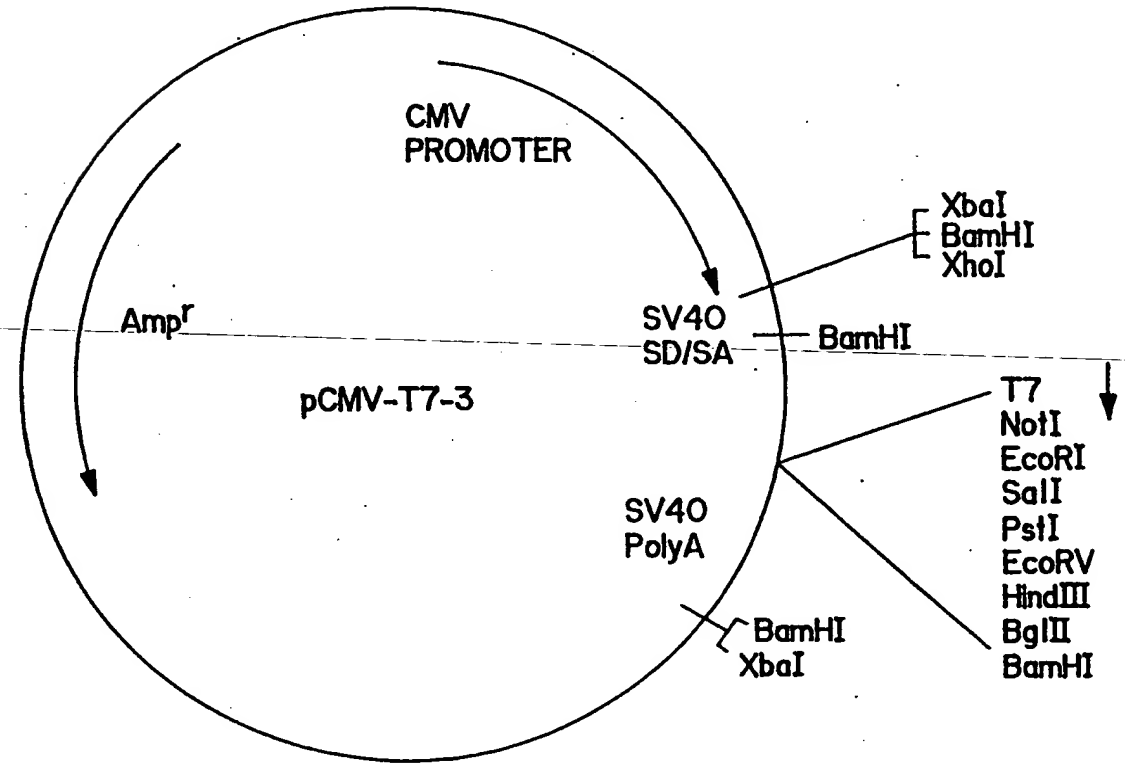
XbaI


CMV
PROMOTER
Amp^r
pCMV-T7-3
SV40
SD/SA
SV40
PolyA
XbaI
BamHI
XhoI
BamHI
T7
NotI
EcoRI
SalI
PstI
EcoRV
HindIII
BglII
BamHI
BamHI
XbaI

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/12859

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :C07K 14/705; C12N 15/00; C12Q 1/00
US CL :435/4, 69.1, 240.1; 530/350; 536/23.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/4, 69.1, 240.1; 530/350; 536/23.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Medline, Dialog, WPI
search terms: nicotinic acetylcholine receptor, human, neuronal, subunits, cloning

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Genomics, Volume 13, issued 1992, Anand et al, "Chromosomal Localization of Seven Neuronal Nicotinic Acetylcholine Receptor Subunit Genes in Humans", pages 962-967, see pages 963 and 964.	1-17
Y	Neuron, Volume 1, issued March 1988, Deneris et al, "Primary Structure and Expression of $\beta 2$: A Novel Subunit of Neuronal Nicotinic Acetylcholine Receptors", pages 45-54, see pages 46 and 47.	7-10, 14, 17
X --- Y	Society for Neuroscience, Abstracts, Volume 16, Part 1, issued 1990, Nash et al , "Molecular Cloning and Expression of Human Neuronal Nicotinic Acetylcholine Receptor Subunits", page 10, Abstract 9.10, see entire abstract.	1-6, 11-13, 15, 16 --- 7-10, 14, 17

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 24 JANUARY 1995	Date of mailing of the international search report 08 FEB 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Sally P. Teng Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/12859

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Science, Volume 240, issued 15 April 1988, Wada et al, "Functional Expression of a New Pharmacological Subtype of Brain Nicotinic Acetylcholine Receptor", pages 330-334, see entire document.	1-17